Description

Process of proportioning of the oestrogens and the androgenes by amplification and bioluminescence.

The proportioning of the oestrone and oestradiol by enzymatic amplification was described by the authors (Patent fran ççais 2 419 519 of INSERM (1), Perfectionnement brought to the processes of proportioning of the oestrogenes and together pret with employment for the realization of the aforesaid proportionings), and Nicolas J.C., BOUSSIOUX A.M., DESCOMPS B, CRASTES of PAULET A.: Covering joint. Chim. Acta, 1979, 32, 1-9 (2) but possede a limited sensitivity (10 pg) and requires a relatively long time of incubation from 5 to 16 hours, to allow the accumulation of a sufficient quantity of NADH which is proportioned by measurement of the absorptance with 340 Nm.

The use of the bioluminescence of marine bacteria of type Beneckea harveyi (Photobacterium fischeri stock MAV) and Vibrio fischeri was the several review object (De Luca Mr. (éed.) - 1978
Methods in Enzymology vol. 57 Academic Presses New York (3) and whitehead T.P., Kricka L.J., Carter T.J.N. and Thorpe G HG: "Analytical Luminescence; its potential in Clinical Laboratory ", Covering joint. Chem.

1979, 25 (9) p. 1531-1546) (4).

More recently, of described proportionings of stéeroidesont, appealing is with the bioluminescence EFord J and De Luca. Analytical biochemistry 1981 - 110 - p. 43/48: has new assay for picomoles levels of androsterone and testosterone using Co-immobilised Luciferase, oxydoreductase and steroid deshydrogenase (52 is with the chemiluminescence gStrasburger CH J, Fricke H. and Wood W.G.: Development and Optimisation of A chemiluminescent Immunoassay (CLIA) Fres. Z.

Anal. Chem. 1982. 311, 351-352 (6) and Klinger W, Van Postel G,
Haupt 0. and Knupper R.: Chemiluminescent Immunoassay of unconjugated
Estriol in Serum of pregnant women - Fres. Z Anal . Chem. 1982, 311, 352-353 (7) and Kim J.B., Barnard G.J., Collins W.P. Kohen F,
Lindner H.R., Eshnar Z: Measurement of Plasma Estradiol 17 ss by solid Phase chemiluminescence immunoassay - Covering joint. Chem. 1982, 28/5, 1120-1124) (8) } but these processes is not very sensitive or owes uti- liser a technique in heterogeneous phase of not very easy employment.

The process of proportioning of the oestrogenes and the androgenes according to the invention is a combination of the enzymatic process of amplification calling upon a specific transhydrogéénase (the 17 } hydroxystéeroide déeshydrogéénase) on the one hand, and, on the other hand, process of proportioning of the NADH formed by bioluminescence bringing into play a chain of oxydoreduction of the NADH and the luciféérase extracted from Beneckae harveyi.

The combination according to the invention makes it possible to ensure the specificity of the system, avoiding the use of a proportioning in phase héétéérogene and giving a great sensitivity which makes it possible to operate with a relatively short time of incubation.

The proportioning of the oestrogenes (oestrone and oestradiol) uses the reaction of transhydrogenation catalysed by the 17 B oestradiol-deshyurogenase of the human placenta which, in the presence of oestradiol and from uestrone, transfers hydrogen from NEDERau NAD, leading to an accumulation of NADH proportional to the concentration in oestrogens (oestrone + oestradiol).

The system of transfer of hydrogen is carried out by the interconversion of the oestradiol oestrone, this reaction being done in less than one second, and, the substrate being constantly recycled, the reaction can be carried out during a time of incubation of a few minutes to several hours, thus involving a factor of amplification ranging between 1000 and 100 000 according to time's of incubation.

The process previously described (references (1) and (2) above) implements like method of proportioning of the NADH a practical but not very significant method, using the absorptance of this coenzyme with 340 Nm, and requires a relatively high factor of amplification, therefore a time of incubation of several hours.

The use of enzymes bioluminescentespermet to proportion quantities of NADH about the picomole and thus, associated the system of transhydrogéénase, to reduce the time of amplification considerably and to increase the sensitivity of proportioning.

The reagent of proportioning of NADH by bioluminescence includes/understands a NADH - FMN oxydoréééductase (FMN = flavin mononucléeotide) which catalyses the reaction
NADH + FMN + H @ NAD + FMNH2 and a luciféérase which uses the produced FMNH2, in presence of an aldehyde with long chain (RCHO) and of oxygen, to produce read mièère according to the following reaction, coupled with the preceding one FMNH2 + RCHO + O2 # FMN + RCOOH + HÒÒ + H #
One measures with the photometer the intensity of produced light, which is proportional to the concentration of NADH.

I - Proportioning of the oestrogens (oestrone + oestradiol) in plasma or the urine
The process according to the invention uses, on the one hand, a plug transhydrogéénase containing the following components: - glucose-6-phosphate = 10-2 to 10-5 mol./l, preferably 10-3 mol./l - glucose-6-phosphate déshydrogéénase = 10 to 1 000 unitées/l and pre- féérence 100 unitées/l - NAD = 10-2 to 10-5 mol./l, preferably 2.10-4 mol./l - NADP = 10-5 to 10-10 mol./l, preferably 2,10-7 mol./l in addition a reagent of bioluminescence containing - FMN = = 0,5 to 10 mg/l, preferably 5 mg/l - aliphatic aldehyde = 0,2 with 20Sug/l, preferably 10 g/l of déécanal - NADH/FMN oxydoréééductase = 2 to 50 U.I./l and, preferably 20 U.I./l - luciféérase extracted from Beneckea harveyi = 1 to 30 mg/l, preferably 20 mg/l
The protocol of proportioning is as follows
One uses 3 tubes (indicated 1,2 and 3) containing each one - 10 to 1 000 L, preferably 200jil of plug transhydrogéénase - and the sample to be proportioned: that is to say 1 to 5 L of urine or 10 to 30 L of plasma.

Tubes 2 and 3 receive 10 to 500 L, and preferably 100 L, (that is to say approximately 10 milliunitées/l) of 17 U - hydroxystééroïïde déshydrogéénase.

Tube 3 receives a known overload, from 5 to 50 pg (preferably 10 oestradiol pg).

The 3 tubes are incubated of 10 min. at 2 hours, each one according to the necessary sensitivity (preferably 15 to 30 minutes).

Then, one sequentially adds to each tube the reagent of bioluminescence and one measures luminescence (variation of the intensity of the light) on 30 seconds or 1 minute.

The calculation of the quantity of oestrone + oestradiol present in the sample is carried out comparing the results obtained for ltechantillon (tube 2 - tube 1) with the results obtained for the overload (tube 3 - tube 2) of known concentration.

It should be noted that it is necessary, immediately before measuring the luminescence of the pilot tube (1) to inject 100 L of 17 ss - hydroxystééroïïde déshydrogéénase, then the reagent of bioluminescence.

Because of the great sensitivity of the method (about the femtomole) the use of antibody anti-steroid can be planned to increase the specificity of the method of proportioning and thus can be applied to the specific proportioning of the oestrone, of oestradiol and the various androgens.

TI - Proportioning specific of oestradiol in the seruin or the urine.

The principle of proportioning rests on the specific absorption of the steroid to proportion by a specific antibody. In the case of oestradiol

- proportioning is carried out on three tubes the witness contains the sample to be proportioned (oestradiol + oestrone) treated beforehand during 30 mn, by one immunoabsorbant specific steroid to be proportioned. Tubes 2 and 3 contain the normal sample and the third tubes an overload known in steroid to proportion The specific proportioning of the oestrone or oestradiol can thus be realiséé directly by addition of the complete reagent of transhydrogéénase, incubation 15 to 30 minutes and proportioning of the NADH by bioluminescence (according to I).

Another simpler way to proportion oestradiol specifically consists in making react the oestrone with a reagent of ketones such as hydrazine. The process is as follows
- the tube "oestradiol + oestrone" contains from 100 to 1 000 L, preferably 500 L, of serum or urine and 100 to preferably 120 L, of distilled water. The tube "oestradiol' T contains the same quantities of serum or urine, but distilled water is rem- placed by an equal volume of hydrazine hydrochlorate 6mol/l pH 7,4.

After drafting, the contents of each tube are extracted by 1 with 5ji1 from ether which is washed by an equal volume of sorting HCY 0,1 mol/l. The separate ééthéérééée phase is evaporated, the residue taken again by 250 L of plug transhydrogéénase is proportioned according to the protocol described to the 3 I.

III-proportioning of the androgens.

The proportioning of the androgens requires the use of an enzyme able to convert the androgens into oestrogens, membrane enzyme contained in the placenta. This enzyme must be removed from its contents in steroid. The purification is led according to the process below. The preparation contains the following activities: a sulfatase, one 3 p - hydroxystééroïïde - dééshydrogéénase, a a5, #4 - céétostééroide - isoméérase and a aromatase. It is indicated hereafter by the term "aromatase".

1. Method of preparation of the aromatase
1/1. Preparation of the microsomes:
The cotyledons of 2 placentas are homogenized in 1 liter of plug phosphates 30mmol/l, pH 7,2, 20 glycerol Z. The homogenate is centrifuged to 10 000 G during 30 mn. The supernatant is again centrifuged 60 mn to 105 000 G the base corresponding to the microsomiale fraction is washed 2 times by 500ml plug. Washing takes place by homogenisation then centrifugation.

1/2. Solubilization of the microsomes
The solubilization of the microsomes must quickly be carried out in order to avoid any loss of enzymatic activity during this stage. The base of microsomes previously obtained is suspended in 50 ml of plug phosphates (30 mmol/l, pH 7,2, 25 glycerol Z), then one adds 50 ml of this same plug containing 8 7 of X100 triton. The suspension is homogenized with 40C in Potter (glass Teflon) during 30 mn. The suspension is then centrifuged 60 mn to 105 000 G.

The X100 Triton is eliminated from this supernatant by chromatography on hydrophobic resin of type XAD2(R).La solution passed on a column (0 2,5 cm X 15 cm) of resin balanced against the plug phosphates. The solution of aromatase is éélueée thanks to this same plug and the coloured fractions are gathered.

1/3. Measure activity aromatase measurement is realized in 350 zip plug 200 mmol/l -5 @@@@@@@@ Tris HCl, pH 8 container 10 mol NADP, 10 mol glucose-6-phosphate, mol dithiothreitol, 0,1 unit glucose-6-phosphate dééshydrogéénase ˜R and 10 5 mol of substrate stééroide (androstèènedione or testosterone or dééhydroéépiandrostéérone). The solution of aromatase is added and it in cubation continued during 15, 30 and 60 mn. The formed oestrogens are proportioned according to the usual process on 100 L of the medium.

2. Proportioning of sulphate of dééhydroéépîîandrostéérone plasma tick (SDHEA) proportioning is carried out on a quantity from 0,5 to 1 L of serum. The plug of transformation of dééhydroéépiandrostéérone (DHEA) into oestradiol same as that is previously described for the measurement of the aromatase activity, i.e., a plug 200 mmol/l Sorting-HCl, pH8. With 60yul of plug containing the aromatase, one adds 40 water L to constitute the tube (1) correspondent with the witness aromatase.

As the quantity of serum used is very weak, proportioning néèces site not of tube corresponding to the witness serum. Optical densities of the tube (2) proportioning - serum (to which one adds 20 L of a dilution of serum + water 20jil) and tube (3) proportioning + overload (to which one-ajou you 20 > it of a dilution of serum + 20 L of plug containing 2p moles of SDHEA) are read, at the end of the proportioning, against the witness aromatase (1).

The quantity of eromatase added under a volame in ral of lOjAl must be sufficient to ensure a total conversion of 10 ng of DHEA. The time of incubation lies between 30 mn and 2 hours.

After aromatization, from 0,2 to 1 ml, preferably 0,2 ml, of plug transhydrogéénase are added, incubation is continued 10 to 30 mn, preferably 10 mn, the tubes are measured sequentially after addition of 100 L of reagent of bioluminescence, in the same way which describes above for the proportioning of the oestrogens.

3. Proportioning of the unit DHEA, #4 - androstèènéédione, testosterone: In order to eliminate from the serum sulphate of DKEA, it was born cessaire to carry out an extraction by the éether1 the sulphate of DHEA not being extractable: 0,5 ml of serum are extracted by 4 ml from ether.

The phase éethéérèée, after washing is evaporated and the residue taken again by 0;5 ml of plug sorting 0,1 mol/l, pH 7,2. 20 L of this extract (sample) are treated by 50 L of plug containing the aromatase according to the following process - the pilot tube (1) contains the inactivated aromatase as well as the sample, - the tube proportioning (2) contains the not-inactivated aromatase as well as the sample, - the tube overloads (3) contains the aromatase non-inac tivéée, the sample and the overload.

After incubation 30 minutes at 37 C, the aromatase is inactivated by heating or alkaline treatment follow-up of neutrali sation. One adds 200 pl then plug transhydrogéénase. A incu bation of 15 mn at 2 hours with 20-250C, according to the wished sensitivity, is followed of addition of 100 ul of reagent of bioluminescence; the measurement of luminescence is made like previously.

4. Testosterone proportioning + androstéénéédione plasmatic proportioning requires the extraction by ether of 1 ml of plasma or the use of a similar technique of extrac tion (column of extréélut). Proportioning can be realizes on the equivalent of 200)11 of plasma according to the preceding process. One needs however uti liser, like system of aromatization, a preparation of which activity 3 - hydroxystééroide dééshydrogéénase of enzymes is inhibited by the 11. - progesterone bromoacéétate (in order not to transform the DHEA).

Inhibition is carried out with 250C, with pH 7,9 by 10 mol/l of 11=bromoacéétoxyprogestéérone (this derivative was synthesized by estéé- rification of the 11.-hydroxyprogestéérone by the bromoacéétate). The sulphate overload of DHEA is replaced by a testosterone overload. The concentration in enzymes must be equal or lower than 100vug/ml.

Inhibition is quasi complete after 2 hours of incubated tion. The excess of steroids is removed by a chromatography on hydrophobic D sine according to the preceding process. The duelle activity aromatase réési measured with the dééhydroéépiandrostéérone must be lower than 5 % of that measured with testosterone.

5. Proportioning of testosterone using the antibodies antitestosterone: Proportioning calls upon three tubes, each one containing 50 L of serum extract corresponding to 5 L of serum. The tube (1) corresponds to the witness serum and will be treated during 15 mn with 250C by 50 L of antibody antitestostéérone. The tube (2) will receive 50 L of sorting. The tube (3) 50 > ul of sorting containing 12 pg testosterone. After addition of 50 1 of plug aromatase and 30 mn of incubation at 37 C, one inactive the aromatase; Then one adds 300 L with plug transhydrogéénase and incubates 1 hour with 370C. After addition of 100 L of reagent of bioluminescence the tubes are read sequentially.

The advantages of the process of proportioning according to the invention are as follows This method, by using at the same time enzymatic amplification and the bioluminescence, makes it possible to carry out

specific proportionings of the oestrogens and androgens with a sensitivity higher than the radioimmunological methods (profit of sensitivity 10) and relatively requires only a time of incubation court Moreover, this method can be automated easily with an already existing instrumentation On the level of specificity, it makes it possible to cumulate at the same time the specificity of the enzyme and-that of the antibody. The use of an internal standard avoids the uses of a curve standard and highlights the interferences due to the mediums.

Claims

1. Proceeded of proportioning of the oestrogens and the androgens present in the biological environments, characterized in that it combines the enzymatic process of amplification using a reaction of transhydrogenation leading to the accumulation of NADH, and the proportioning of the NADH formed by bioluminescence.

2. Proceeded according to claim 1, characterized in that one associates the 17 ss - hydroxystééroïïde dééshydrogèènase of human placenta for the reaction of transhydrogenation, in Nadh-fmn oxydorééductase and a lucifééerase extracted from micro-organisms for proportioning by bioluminescence, in the presence of the coenzymes and-aldehyde necessary.

3. Proceeded according to one of the claims 1 and 2, characterized in that one uses a reagent of transhydrogenation or "plug transhydrogèènase" consisted:
- 10-2 to 10-5 glucose-6-phosphate mol/l
- 10 to 1000 glucose-6-phosphate dééshydrogèènase unitéés/l
- 10-2 to 10 mol/l of NAD
- 10 5 to 10 10 mol/l of NADP 4. Proceeded according to one of claims 1 àà3, characterized in that one uses a reagent of bioluminescence consisted
- 0,5 to 10 mg/l of FMN
- 0,2 A 20iug/l of an aliphatic aldehyde such as the déécanal
- 2 to 50 UI/l of NADH/FMN oxydoréédueétase
- 1 to 30 mg/l of lucifééerase of Beneckea Harveyi 5.Procéédéé according to one of claims 1 to 4, characterized in that one proportions the couple oestradiol-oestrone in the blood plasma or the urine by using 3 test tubes containing each one Echan- tillon to be proportioned and the plug - transhydrogenase; tube 1 is the pilot tube and tubes 2 and 3 receive each one of the 17ss-hydroxystééroïïde- dééshydrogèènase; tube 3 receives, moreover, one known oestradiol overload; after incubation of the tubes of 10 mn at 2 hours, one adds to each tube the reagent of bioluminescence and measurement luminescence over 30 seconds or 1 minute; one calculates the quantity of oestradiol + oestrone in the sample by comparing the results obtained for the sample (tube 2 - tube 1) with the results obtained for the overload (tube 3-tube 2) of known concentration.

6. Process according to one of claims 1 to 5, characterized in that one proportions specifically oestradiol or the oestrone in plasma or the urine by initially treating the sample by one immunoadsorbant specific steroid to be proportioned, then by proportioning the sterolde by addition of the reagent of transhydrogèènase, incubation and proportioning of
NADH by bioluminescence.

7. Process according to one of claims 1 to 5, characterized in that one proportions specifically oestradiol in plasma or the urine by initially eliminating ltoestrone by reaction with lthydrazine.

8. Proceeded of proportioning of the androgens according to one of claims 1 to 4 characterized in that one converts initially the androgèè- nes into oestrogens by action of an enzyme extracted the placenta and which presents activities of sulfatase, of 3 ss-hydroxystééroïïde-dééshydrogèè- nase, de#5, #4-céétostééroïïde-isoméérase and of aromatase, and that one proportions then the oestrogens, after having inactivated the aromatase by heating or alkaline treatment, by addition of the reagent of transhydrogèènase, incubation and proportioning of the NADH by bioluminescence.

9. Proceeded of proportioning of the couple testosterone + androstèènéé- dione according to claim 8, characterized in that one uses, for the conversion of the androgens into oestrogens, an enzymatic preparation of which activity 3 ss-hydroxystééroïïde - dééshydrogèènase is inhibited by the 11-bromacéétate progesterone.